



The initial tolerance to sub-lethal Cd exposure is the same among ten naïve pond populations of *Daphnia magna*, but their micro-evolutionary potential to develop resistance is very different



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ABSTRACT

Genetic variation complicates predictions of both the initial tolerance and the long-term (micro-evolutionary) response of natural *Daphnia* populations to chemical stressors from results of standard single-clone laboratory ecotoxicity tests. In order to investigate possible solutions to this problem, we aimed to compare the initial sub-lethal tolerance to Cd of 10 naïve natural pond populations of *Daphnia magna* as well as their evolutionary potential to develop increased resistance. We did so by measuring reproductive performance of 120 clones, i.e. 12 clones hatched from the recent dormant egg bank of each of 10 populations, both in absence (Cd-free control) and presence of 4.4 µg Cd/L. We show that the *initial tolerance*, defined as the reproductive performance of individuals of the first generation exposed to Cd relative to that in a Cd-free control was not significantly different among the 10 studied pond populations and averaged 0.82 ± 0.04 over these populations. Moreover, these populations' initial tolerances were also not significantly different from the mean initial tolerance of 0.87 ± 0.08 at 4.0 µg Cd/L measured for a group of 7 often-used laboratory clones, collected from a range of European ecotoxicity testing laboratories. This indicates that the initial response of naïve natural pond populations to sub-lethal Cd can be relatively accurately predicted from ecotoxicity test data from only a handful of laboratory clones. We then used estimates of broad-sense heritability of Cd tolerance (H^2) – based on the same dataset – as a proxy of these populations' capacities to evolutionarily respond to Cd in terms of the development of increased *resistance*, which is here defined as the increase with time of the frequency of clones with a higher Cd tolerance in the population (accompanied with an increase of mean Cd-tolerance of the population above the initial tolerance). We show that the populations' estimated H^2 values of Cd-tolerance cover almost the entire theoretically possible range, ranging from not significantly different from zero (for five populations) to between 0.48 and 0.81 (for the five other populations). This indicates that, unlike the initial tolerance to Cd, the (long-term) micro-evolutionary response to Cd may be very different among natural pond populations. Therefore, we conclude that it may be very difficult to predict the long-term response of an unstudied population to chemical stress from tolerance data on a sample of other populations. It is therefore suggested that new methods for forecasting long-term responses should be explored, such as the development of predictive models based on the combination of population-genomic and tolerance time-series data.

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1. Introduction

Extrapolating results of laboratory ecotoxicity tests to predict effects of chemicals on natural populations in the field remains one of the big challenges in ecotoxicology and risk assessment (Forbes

et al., 2001; Vandenbrink, 2008). One reason for this is that standard ecotoxicity tests are often conducted with laboratory populations, which often harbor little genetic diversity (Medina et al., 2007; Barata et al., 1998). This is for instance the case with *Daphnia magna*, which is one of the most sensitive and most frequently used species in aquatic ecotoxicity testing (Von der Ohe and Liess, 2004). Indeed, most ecotoxicity tests with *D. magna* and other *Daphnia* spp. are performed with isoclonal populations of female individuals that are maintained in a laboratory culture through parthenogenetic

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reproduction. This situation contrasts profoundly with the fact that, in the field, *Daphnia* populations can be genetically highly diverse (De Meester et al., 2006). This contrast brings about two important problems for predicting the response of a naïve (i.e., previously unexposed) natural *D. magna* population that is challenged with a chemical stressor (Medina et al., 2007; Morgan et al., 2007; Klerks et al., 2011).

First, an ecotoxicity test with a single laboratory clone will usually not be predictive of the *initial* (i.e., *proximate*) response of a natural *Daphnia* population consisting of many genetically different clones (Barata et al., 1998, 2002a), with the *initial* response being defined throughout our paper as the effect of the chemical (relative to a control) on the individuals of the first exposed generation.

Second, under multi-generational exposure, genetically diverse natural *Daphnia* populations may exhibit natural selection of more tolerant clones, i.e. those clones that experience a smaller adverse effect of the chemical (relative to a control) (Ward and Robinson, 2005; Lopes et al., 2006). This may eventually result in selection-mediated increased *resistance* of the natural population. Increased *resistance* is defined throughout our paper as an increased frequency of occurrence in the population of clones with a higher tolerance to the chemical (i.e., *genetic adaptation*; Morgan et al., 2007). This is a micro-evolutionary response that cannot be predicted from single-generation ecotoxicity tests with a single laboratory clone.

A possible solution to these two problems would be to be able to make predictions of initial and micro-evolutionary responses based on tolerances determined for a set of clones isolated from a set of naïve natural populations. Indeed, some studies are suggestive that such an approach would enable at least some broad predictions.

First, (Hoffman and Parsons, 1991, 1997) postulated that differences in tolerance to toxicant stress among populations should reflect local genetic adaptation to selective pressures experienced in their local habitats. Populations from distinct habitats with no history of pollution with a toxicant (i.e. *naïve* populations) are therefore expected to show similar levels of tolerance to that toxicant. This has been confirmed by Barata et al. (2002a, 2002b), who reported similar reproductive effects of sub-lethal exposure of natural *D. magna* populations to an insecticide (λ -cyhalotrin) and a metal (Cd) in a study involving three and four populations, respectively. Thus, extrapolating initial responses to chemicals from one natural population to another may provide reasonably accurate predictions.

Second, given that (i) standing genetic variation (e.g., when measured as heritability) of chemical tolerance within a natural population is considered a valuable proxy of the micro-evolutionary potential and may be used to calculate the rate of adaptation to chemical stress through natural selection (Chaumot et al., 2009; Klerks et al., 2011; Messiaen et al., 2012) and (ii) that the heritability of many traits is often similar among different populations of the same species (Visscher et al., 2008), the micro-evolutionary response of naïve natural populations to chemical stress may indeed be sufficiently similar to extrapolate observations with one population to other populations. Initial support for this has been provided by Barata et al. (2002b), who reported similar broad-sense heritability values (H^2) of sub-lethal (reproductive) Cd tolerance in two Cd-naïve *D. magna* populations.

In the present study we aimed to build further on the earlier work of Barata et al. (2002a,b), also using Cd as the model chemical, but using a much larger set of 10 naïve natural *D. magna* pond populations in order to address two main questions: (i) Is the *initial* sub-lethal tolerance to a nominal concentration of 5 $\mu\text{g Cd/L}$ (expressed as reproductive performance relative to a control) the same or different among 10 natural populations (i.e., is the *proximate* effect of a standardized exposure to a chemical similar or different among populations)?, and (ii) Is the evolutionary potential

(based on measurements of broad-sense heritability as its proxy) of sub-lethal Cd tolerance similar or different in these populations? The investigated populations were established from the dormant, ephippial egg bank from a broad variety of habitats in terms of their abiotic and biotic characteristics, and have been shown to differ substantially in genetic composition (Orsini et al., 2012). They were also confirmed to be naïve with respect to Cd exposure in the sense that all habitats are characterized by low to very low concentrations of Cd (Table 1).

In order to be able to discuss our observations in a broader regulatory context, we chose to perform our experiments at a regulatorily relevant low sub-lethal effect level, by exposing the natural populations to a Cd-concentration close to the geometric mean of the 21-day 10% effective concentration based on reproductive performance (21d-EC₁₀, see Section 2 for details) of 7 laboratory clones, collected from 7 different ecotoxicity testing laboratories across Europe. The geometric mean of multiple chronic (or sub-lethal) EC₁₀s for the same species is often used in EU chemicals legislation as a basis for derivation of predicted no effect concentrations (PNEC) or environmental quality standards (EQS) of chemicals (ECHA, 2008; EC, 2011a). The results from these laboratory clones also provide information on the range of tolerances to a model toxicant among often-used laboratory clones.

2. Materials and methods

2.1. Seven *D. magna* laboratory clones

Several individuals from 7 different *D. magna* laboratory clones were shipped from different ecotoxicity testing laboratories across Europe to the UGent laboratory (see *Supportive Information*, Table S1, for details of the origin of the clones). One individual of each clone was randomly picked out to establish our own in-house isoclonal cultures of these clones. These in-house cultures were first established in April 2009, which allowed the clones ample time to acclimate to our in-house culture conditions (see further) before actual experiments started in October 2009.

2.2. Clones from ten natural *D. magna* populations

The recent dormant egg bank (ephippial eggs) of 10 pond populations in Flanders (Belgium) was sampled between January and March 2007 by collecting the upper 2 cm of the sediment using a sediment corer. The genetic diversity of the dormant egg bank of *Daphnia* spp. at the sediment surface layer is commonly considered to be representative of the genetic diversity of the actual (live) population that is established by hatching of ephippial eggs at the start of a new growing season (e.g., Wolf and Carvalho, 1989; Antunes et al., 2003; Orsini et al., 2012).

Seven ponds were located in the province of Flemish-Brabant (near Leuven) and three ponds were located in the province of Western Flanders (near Knokke). Details on location and characteristics of each pond are provided in Table 1 and as *Supportive Information* (Table S2).

The investigated ponds represented a broad variety of habitats in terms of their biotic and abiotic characteristics (Orsini et al., 2012). In addition to those characteristics, we also sampled water and sediment from the ponds to determine metal concentrations, to ensure that the ponds were not contaminated with Cd (or other metals) (see Section 2.5, for analytical methods). Comparison of Cd measured in water and sediment with natural background Cd concentrations confirmed the absence of Cd pollution in all ponds (Table 1), which was already expected based on the absence of important sources of Cd in the vicinity of the ponds. Furthermore Cd concentrations in the water are in all ponds below the hardness-corrected EQS values (derived following EC, 2005), meaning that no

Table 1

Overview of natural pond populations and some habitat characteristics.

Pond ID	Lat (N)	Long (E)	Hardness (mg CaCO ₃ /L)	EQS (dissolved Cd) ^a (μg/L)	Total Cd in H ₂ O (μg/L)	Total Cd/ dissolved EQS	Total Cd/ total bgc ^b	Cd in sediment (mg kg ⁻¹ dry wt)	Cd (sed)/ bgsc ^c
KNO15	51°20'05.52"	03°20'53.63"	158	0.211	0.061	0.29	0.15	1.2	0.39
KNO17	51°21'01.97"	03°19'49.58"	286	0.328	0.063	0.19	0.15	1.8	0.60
KNO52	51°20'11.27"	03°20'55.31"	201	0.252	0.059	0.23	0.14	1.4	0.46
LRV	50°49'42.08"	04°38'20.60"	185	0.237	0.012	0.05	0.03	3.3	1.05
OHZ	50°50'22.09"	04°39'18.16"	142	0.195	0.042	0.22	0.10	1.2	0.40
OM2	50°51'47.82"	04°43'05.16"	132	0.185	0.058	0.32	0.14	2.3	0.74
OM3	50°51'47.32"	04°43'05.16"	184	0.236	0.018	0.08	0.04	1.1	0.37
TER1	50°49'22.98"	04°35'38.17"	74	0.120	0.099	0.83	0.24	1.5	0.48
TER2	50°49'18.24"	04°36'04.50"	242	0.290	0.086	0.30	0.21	1.6	0.52
ZW4	50°49'24.68"	04°39'53.46"	235	0.283	0.012	0.04	0.03	2.5	0.81

^a Cd EQS = 0.09 × (Hardness/50)^{0.7409} (EC, 2005).^b Total bgc = 90th percentile of Cd natural background concentration; values have been reported for unpolluted freshwater in Netherlands = 0.41 μg/L (total Cd) (Crommentuijn et al., 1997) and for Northern European lowlands = 0.78 μg/L (total Cd) (Zuurdeeg, 1992); the calculated ratio is based on the bgc of 0.41 μg Cd/L.^c bgsc is based on mean + 1S.D. of background sediment Cd concentration in Belgian freshwater sediments (mean = 1.6 mg/kg, S.D. = 1.5 mg/kg) = 3.1 mg/kg (Swennen et al., 1998).

adverse effect of Cd on ecological structure and function is expected in either of the ponds (Table 1). Comparison of other metal concentrations (Zn, Ni, Cu, Pb) in water and sediment with background concentrations and with dissolved organic carbon (DOC) normalized HC5 values (hazardous concentration for 5% of the species) for Zn, Ni, Cu (Zwolsman and De Schampelaere, 2007) or with EQS for Pb (EC, 2011b) suggests very limited to no metal pollution and no metal-induced ecological effects in any of the ponds (See *Supportive Information*, Table S2). In addition, total Cd concentrations in water (0.012–0.099 μg/L, Table 1) are well below the geometric mean hardness-corrected 21d-EC10 for laboratory clones (0.8–11.2 μg/L, Table 2), suggesting that the current (low) Cd exposure in the ponds is expected not to affect *D. magna* reproductive performance in the ponds.

Cladoceran dormant eggs were isolated by means of the sugar flotation method (Onbe, 1978; Mareus, 1990). Briefly, sediment was transferred together with an oversaturated sugar solution (1000 g sugar in 1000 mL of distilled water) into 50 mL Falcon tubes. These tubes were centrifuged (10 min at 3000 × g) and decanted twice. Most ephippial eggs then floated in the decanted sugar solution and were easily isolated. The remaining sediment in the tubes was inspected visually and any remaining dormant eggs were picked out manually. All isolated *D. magna* eggs were put individually in ADaM medium (Aachener Daphnien Medium; Klüttgen et al., 1994) in a climate room at 20 °C and under a 16-h:8-h light:dark photoperiod. Medium was refreshed every 8–9 days and hatchlings were isolated daily. A single hatchling from each ephippium was selected to establish a clonal lineage (Ebert et al., 1993). As dormant eggs of *D. magna* are produced by sexual reproduction, each clonal lineage hatched from an ephippium can be considered genetically distinct (Barata et al., 2000a,b). Clonal lineages were first maintained at KULeuven in 300 mL of tap water and were fed two times a week with 10⁸ cells of *Scenedesmus obliquus*. In December 2008,

twelve randomly selected clones from each of the ten natural pond populations (120 clones in total) were transported to UGent for establishing in-house cultures of these clones, which were maintained until the actual experiments.

2.3. Maintenance cultures of *D. magna* clones

The 7 laboratory clones and the 120 clones hatched from dormant egg banks were maintained at 20 °C and under a light:dark photoperiod of 16-h:8-h. The culture medium was a modified M4 medium, which is different from the original composition of M4 medium (Elendt and Bias, 1990) as follows: Na₂EDTA and FeSO₄ were omitted and replaced with natural dissolved organic matter (DOM). The DOM was collected from a small creek (Ruisseau de St. Martin, Bihain, Belgium) using a portable reverse osmosis system (PROS/2) (Sun et al., 1995). This modified M4 medium has a hardness of 250 mgCaCO₃/L, a pH of 7.6, and a concentration of dissolved organic carbon (DOC) of 4 mg/L. Individuals of each clone were kept in polyethylene vessels in 50 mL of M4 medium. Once every week, 1 or 2 juveniles and 1 or 2 adults (daphnids carrying parthenogenetic eggs) of each clone were transferred to fresh medium. Each clone was fed daily with a 3:1 mixture (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*, at an amount of 500 μg dry wt per day and per 50 mL. Maintenance of all clones continued in this way until October 2009, when the actual experiment was initiated.

2.4. Experimental design

The experiments with the laboratory clones and the clones hatched from dormant egg banks were identical with only two exceptions. First, while each laboratory clone was exposed to 6 treatments (5 Cd concentrations and a control), only two

Table 2The 21-day 10% effect concentrations (EC₁₀) of Cd, the intrinsic rate of increase (*r_m*) under control (no Cd added) and 4.0 μg Cd/L, and Cd tolerance (*r_m*@Cd/*r_m*@control) for 7 isoclonal laboratory populations as shown by. See supportive information (Table S1) for origin of laboratory clones.

Laboratory clone	EC ₁₀ (μg/L) (95% C.I.)	<i>r_m</i> @ control ± S.D.	<i>r_m</i> @ 4 μg Cd/L ± S.D.	Tolerance @ 4.0 μg Cd/L ± S.D.
CZ	7.8 (5.3–10.0)	0.39 ± 0.02	0.37 ± 0.03	0.94 ± 0.09
Clone K6	0.8 (0.6–1.1)	0.38 ± 0.02	0.28 ± 0.03	0.74 ± 0.07
SE	11.2 (9.2–13.0)	0.40 ± 0.02	0.39 ± 0.02	0.96 ± 0.09
DK	3.9 (3.4–4.5)	0.38 ± 0.04	0.34 ± 0.01	0.92 ± 0.08
Clone F	3.2 (2.5–4.1)	0.37 ± 0.03	0.31 ± 0.04	0.86 ± 0.18
Clone A	2.0 (1.9–2.1)	0.36 ± 0.01	0.28 ± 0.03	0.78 ± 0.06
Clone IRCHA-5	4.9 (4.9–5.1)	0.36 ± 0.01	0.32 ± 0.01	0.90 ± 0.04
Geometric mean	3.7			
Mean ± S.D.				0.87 ± 0.08

EC₁₀ = 10% effective concentration, 95% C.I. = 95% confidence interval, S.D. = standard deviation.

treatments (1 Cd concentration and a control) were imposed on the clones hatched from dormant egg banks. Second, while 6 individuals of each laboratory clone were tested in each treatment, only 3 individuals were used in each treatment for each clone hatched from natural dormant egg banks.

From each clonal lineage, a single, randomly selected third- or fourth-brood juvenile (<24 h old) from the maintenance culture was put individually in 50 mL of modified M4 medium without added Cd as the F0 grandmother generation. Following maturation of this individual, six (for the laboratory clones) or three (for the clones hatched from dormant eggs) of its third- or fourth-brood offspring (<24 h old) were put individually in 50 mL of modified M4 medium without added Cd and were allowed to mature to F1-mothers. From each of these mother individuals, one third- or fourth-brood juvenile (<24 h old) (=F2 experimental generation) was randomly assigned to one of the six (laboratory clones) or two treatments (natural population clones). As such, every replicate in each treatment was represented by a single F2-individual, produced by a different F1-mother. In this way we minimized interference from maternal effects that would otherwise potentially inflate our estimates of genetic variance of traits within populations (Lynch and Walsh, 1998; Messiaen et al., 2012) (see also Section 2.5.5).

Life-table experiments with all F2-individuals, clones and treatments were initiated simultaneously to avoid temporal effects. All exposures were conducted in modified M4 medium (one individual per test vessel) at 20°C and under a 16-h:8-h light:dark photoperiod. Laboratory clones were investigated in six treatments, a control (no Cd added) and nominal Cd concentrations of 1, 2.2, 4.6, 10 and 22 µg Cd/L. Clones hatched from dormant egg banks were investigated in two treatments, a control and a nominal Cd concentration of 4.6 µg Cd/L. The latter concentration was chosen with regard to the aim of the present study to be able to interpret our observations in a broader regulatory context (see Section 1), because preliminary experiments had indicated that this concentration was close to the concentration that caused an average 10% reduction of reproductive performance in the 7 laboratory clones. The modified M4 medium was always spiked with the desired Cd concentration (added as CdCl₂·H₂O) 24–48 h prior to transfer of the daphnids into the medium.

Throughout the entire experiment (P, F1, and F2 generation), organisms were fed daily with a 3:1 mixture (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* equivalent to 250 µg dry wt/*Daphnia*, 500 µg dry wt/*Daphnia* and 750 µg dry wt/*Daphnia* in the first, second and third week of their life, respectively. The medium was renewed completely three times a week (Monday, Wednesday, Friday).

Each individual of the F2-generation was monitored during the life-table experiment for 21 days. Survival and the number of juvenile offspring were recorded daily. During the life-table experiment, samples of new (fresh) and old medium were taken at regular intervals for analysis of filtered (0.45 µm) Cd (using graphite-furnace AAS) and DOC concentrations (using Shimadzu TOC analyzer).

2.5. Data analysis

2.5.1. Intrinsic rates of increase

Intrinsic rates of increase (r_m) were calculated per individual replicate from age-specific fecundities recorded during the life-table experiment, by fulfilling the condition (Caswell, 2001):

$$\sum_{x=1}^{21} F_x e^{-r_m x} = 1 \quad (1)$$

where x is the number of days since the start of the life-table experiment and F_x is the age-specific fecundity (i.e. number of live offspring recorded on day x). Replicates holding a male F2 individual or in which no reproduction occurred (mostly due to parent mortality) were excluded from analysis, as the r_m equals $-\infty$ in these cases. An r_m calculated in this way for *Daphnia* spp. was called 'reproductive performance' by Jansen et al. (2011) and Van Doorslaer et al. (2009). We choose to work with r_m because this is considered a relevant measure of fitness in parthenogenetically reproducing *Daphnia* spp. populations, at least under non-limiting conditions (Lynch and Walsh, 1998; Hooper et al., 2008).

2.5.2. Tolerance

Since F2 individuals from each clone in each Cd treatment always had a 'sister' that was exposed to the control (i.e. they shared the same F1 mother, see Section 2.4), we were able to determine replicate "observations" of tolerance for a given Cd treatment based on r_m values for such 'sister' pairs, of which one was exposed to Cd and the other to the control:

$$\text{Tolerance}(\text{Cd}) = \frac{r_m(\text{Cd})}{r_m(\text{control})} \quad (2)$$

As such, for each Cd treatment, a number of tolerance "observations" was available equal to the number of replicates per treatment (and equal to the number of F1 mothers used per clone, see Section 2.4).

2.5.3. Further data analysis with results of laboratory clones

All analyses started from calculated tolerances (see Eq. (2)). Second-order polynomial regression was used to calculate 21d-EC₁₀ values for each clone (Barata et al., 2002b), except for the K6 clone where a linear regression was more appropriate. Averages of measured dissolved Cd concentrations were used as the independent variable; observed tolerances as the dependent variable. The jack-knife method was used to estimate approximate 95% confidence limits. The regression analyses were performed with Statistica 7 software (Statsoft, Tulsa, OK, USA).

2.5.4. Further data analysis with natural populations (population means)

To test the hypothesis that naïve populations have similar mean (initial) proximate tolerances, we compared Cd tolerances among populations. To this end, we first calculated the clone-mean r_m and Cd tolerance for each clone (as the mean of three replicate observations). All further statistical analyses and comparisons were performed using these clone means as the dependent variables. In order to test if exposure to 4.6 µg Cd/L had a significant initial (proximate) effect on each of the 10 natural populations (compared to the control), we performed a t -test for dependent samples ($p < 0.05$). In case the normality assumption was not met (Shapiro–Wilkinson W, $p < 0.05$), the non-parametric alternative, i.e. the Wilcoxon matched pairs test, was conducted. The Cd-tolerance was statistically compared among the ten populations with the Kruskal–Wallis test ($p < 0.05$).

2.5.5. Further data analysis with natural populations (heritabilities and evolutionary potential)

Heritability, a simple dimensionless measure of the importance of genetic factors in phenotypic differences between individuals, enables predictions about the response to selection in populations and can be compared among populations (Visscher et al., 2008). As *Daphnia* reproduce asexually (parthenogenetically) most of the year, clonal selection is a strong factor in *Daphnia* micro-evolution and broad-sense heritability (H^2) is an appropriate parameter to determine the potential short-term response to (clonal) selection in natural populations (Ebert et al., 1998; Stirling and Roff, 2000).

High heritability of tolerance traits in *Daphnia* predicts the capacity for rapid evolution by clonal selection (Messiaen et al., 2012). Because both fitness (reproduction, survival) under chemical stress itself (e.g. Messiaen et al., 2012; Chaumot et al., 2009; Klerks and Moreau, 2001), as well as relative tolerance (defined as relative fitness under chemical stress compared to a control, see Eq. (2), as in Barata et al., 2002b) have been put forward as useful tolerance traits for predicting evolutionary potential, we considered both in the present study. Relative tolerance as defined here is equivalent to a slope of a reaction norm (Stirling and Roff, 2000) and heritability of relative tolerance as defined here is therefore equivalent to heritability of plasticity, reflected in a significant genotype by environment interaction (Stirling and Roff, 2000). Replicate observations of r_m of all clones were used to determine broad sense heritability (H^2) of $r_m(\text{control})$, $r_m(\text{Cd})$ and of Cd-tolerance following Messiaen et al. (2010). For each population, the genetic variance (V_G) and the environmental (or residual) variance (V_E) were estimated from the observed among-clone (MS_C) and within-clone mean squares (MS_E), using the method of the moments with appropriate accounting for unequal sample sizes among clones, as follows (Table 18.1 in Lynch and Walsh, 1998):

$$MS_C = \frac{\left[\sum_{i=1}^N n_i (\bar{z}_i - \bar{z})^2 \right]}{(N - 1)} \quad (3)$$

$$V_E = MS_E = \frac{\left[\sum_{i=1}^N \sum_{j=1}^{n_i} (z_{ij} - \bar{z}_i)^2 \right]}{(T - N)} \quad (4)$$

$$V_G = \frac{(MS_C - MS_E)}{n_0} \quad (5)$$

$$n_0 = \frac{\left[T - (\sum_{i=1}^N n_i^2) \right]}{(N - 1)} \quad (6)$$

where T is the total number of observations for the population, N the number of clones studied for the population, n_i the number of replicates for the i th clone, z_{ij} the observed value for the j th replicate of the i th clone, \bar{z}_i the mean of all (n_i) observed values for the i th clone, \bar{z} the mean of all (T) observed values for the population, and n_0 is a weighted number of replicates per clone to account for unequal sample size among clones in the calculation of V_G (Searle et al., 1992).

H^2 was calculated as $V_G/(V_G + V_E)$. Construction of confidence intervals and hypothesis testing was performed using non-parametric bootstrap re-sampling (5000 samples) with replacement of clones (Lynch and Walsh, 1998; Messiaen et al., 2010). The median values (50th percentile) and the 5th and 95th percentile of H^2 are reported for $r_m(\text{control})$, $r_m(\text{Cd})$ and for tolerance. Statistical tests were then constructed using the bootstrap output. First, if more than 95% of the bootstrap samples yielded an $H^2 > 0$ (equivalent to a one-sided test at $p < 0.05$ level), we considered that there was an H^2 significantly > 0 , reflecting significant evolutionary potential. Second, if more than 95% of the bootstrap samples yielded $H^2(\text{Cd}) > H^2(\text{control})$ (equivalent to a one-sided test at $p < 0.05$ level), we considered the H^2 in the Cd treatment to be statistically significantly higher than in the control. Third, when more than 97.5% of the bootstrap samples yielded a higher (or lower) H^2 for one population than for another, those two populations were considered to have a statistically different H^2 value for the trait considered (equivalent to a two-sided test at $p < 0.05$ level). All calculations were performed in MATLAB 7.5.0.342 software (Mathworks Inc.).

It has been argued that the interpretation of H^2 is complicated by the fact that it depends both on genetic variance (V_G)

and environmental (residual) variance (V_E) in the observations, as $H^2 = V_G/(V_G + V_E)$ (Klerks et al., 2011). Likewise, it has also been argued that contaminant-driven genetic erosion by directional selection is more likely in populations with a combination of high V_G and low V_E of tolerance to the contaminant (Ribeiro and Lopes, 2013). However, in our study, when considering the results of all populations together, H^2 is strongly correlated with V_G and not with V_E (Figure S12) indicating that any differences found in H^2 among populations mainly have a genetic cause and are not an artifact of uncontrolled differences in environment between replicates or residual experimental error. For this reason, we only report and discuss H^2 (and not V_G or V_E) in the present paper.

2.6. Metal concentrations in water and sediment of the study ponds

In addition to those pond characteristics already recorded and reported previously (Orsini et al., 2012), we sampled water and sediment from the ponds in April 2009 to determine metal concentrations. The upper layer (approximately 10 cm) of the sediment was sampled to determine Ni, Cu, Pb, Zn and Cd. Sediment was acid-digested with the aid of a microwave oven. Ni, Cd, Cu, Zn and Pb were analyzed using flame AAS (Spectra AA 100-Varian) or a graphite furnace AAS (Zeeman, Spectra AA300-Varian). To determine Cu, Ni, Pb, Cd, Na, Ca and Mg concentrations in the water, triplicate samples of 50 mL were collected into Falcon tubes, which had been acid washed and rinsed three times with pond water at each location. Samples were centrifuged for 15 min at $2000 \times g$ in the lab (Centra 8, Thermolife Sciences, Belgolab) and the total concentration of Cu, Ni, Pb, Cd, Ca and Mg in the supernatant were measured with ICP-MS (inductive coupled plasma mass spectrometry, Perkin-Elmer Elan DRC-e, Wellesley, MA, USA).

3. Results

3.1. Cd tolerance of seven laboratory clones

Details of the chemical analyses in the exposures of the seven laboratory clones are reported as *Supportive Information* (Table S3). Across all Cd treatments (including the control), pH was on average 7.7 (range 7.6–7.9) and DOC was on average 5.3 mg/L (range 4.6–6.0 mg/L). In fresh medium, measured dissolved Cd was between 84% and 90% of the nominal concentration. Measured dissolved Cd in old medium was 4–31% lower than in fresh medium. At the nominal Cd concentration of 4.6 $\mu\text{g/L}$, the mean dissolved Cd concentration was 4.0 $\mu\text{g/L}$.

Table 2 shows the differences in Cd tolerance among the seven laboratory clones. Detailed concentration response data and fitted concentration response models are presented as *Supportive Information* (Figure S1). The 21d- EC_{10} s for the laboratory clones varied 14-fold between 0.8 (clone K6) and 11.2 $\mu\text{g/L}$ (clone SE), with a geometric mean of 3.7 $\mu\text{g/L}$. In the 4.0 $\mu\text{g Cd/L}$ treatment, i.e. within less than 10% of the geometric mean EC_{10} of 3.7 $\mu\text{g Cd/L}$, the observed Cd tolerance varied between 0.74 (Clone K6) and 0.96 (clone SE), with a mean of 0.87 (S.D. 0.08, $n = 7$).

3.2. Fitness and Cd tolerance of 10 field populations

Details of the chemical analyses made during the exposures of the 10 field populations are reported as *Supportive Information* (Table S4). The pH was on average 7.7 (range 7.6–7.8) and DOC was on average 5.7 mg/L (range 4.0–7.6 mg/L). At the nominal Cd concentration of 4.6 $\mu\text{g/L}$, the mean dissolved Cd concentration was 4.4 $\mu\text{g/L}$. Measured dissolved Cd in old medium was 9% lower than in fresh medium.

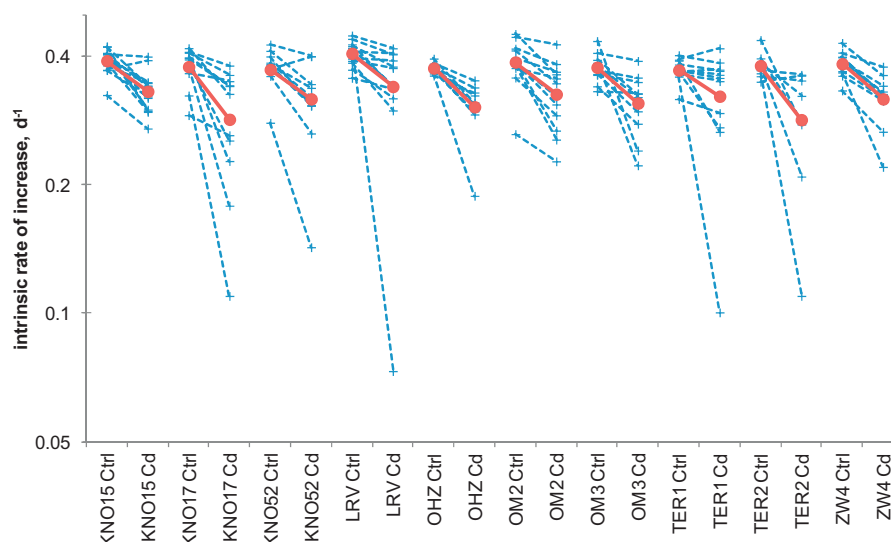


Fig. 1. Reaction norms for the different clones (crosses, dashed lines) and population reaction norms (thicker dots, full lines) for 10 natural *D. magna* pond populations exposed to a control (Ctrl) and Cd (4.4 $\mu\text{g Cd/L}$). Blue crosses represent the mean phenotypes of individual clones within a population (clone means). Red dots represent the population mean (mean of clone means). Values for population means are available in *Supportive Information* (Table S5). Note the (\log_2 -based) logarithmic vertical axis. A steeper slope corresponds with a lower tolerance as defined in Eq. (2) and as shown in Fig. 2.

Fig. 1 depicts the mean r_m under the control ($<0.1 \mu\text{g Cd/L}$) and the Cd environment (4.4 $\mu\text{g Cd/L}$) of each individual clone within each population (reaction norms) and also each population mean (mean of clone means). Fig. 2 depicts the clone means and population means of Cd tolerance. Table S5 in *Supportive Information* provides the population mean values corresponding with these figures. The r_m were significantly lower at 4.4 $\mu\text{g Cd/L}$ than in the control for each of the 10 populations, using a paired analysis and using clone identity to pair data ($p < 0.05$ by t -test for dependent samples or Wilcoxon Matched Pairs test, details in *Supportive Information*, Table S6). The population means of Cd tolerance were all between 0.75 (KNO17 and TER2) and 0.87 (TER1) (average \pm S.D.: 0.82 ± 0.04), which corresponds to a reduction of the r_m between 13% and 25% (Fig. 2, Table S5).

Across all populations, observed tolerances per clone ranged between 0.17 (most sensitive clone in LRV) and 1.13 (least sensitive clone in KNO52) (Fig. 2). Cd tolerances (clone means) were not normally distributed in 5 of 10 populations (i.e., in LRV, OHZ, OM3, TER1 and ZW4; see *Supportive Information*, Table S7) (Shapiro–Wilkinson W, $p < 0.05$). As the null-hypothesis of homoscedasticity (equal variances) was not rejected (Levene, $p > 0.05$), but as none of the classic transformations (logarithmic, inverse, square) were able to

remediate the non-normality issue, an appropriate non-parametric test under these conditions to test for differences in tolerance among populations is the Kruskal–Wallis test. This test did not reveal any significant differences of the mean population tolerance among the populations ($n = 106$, $\text{df} = 9$, $p = 0.715$). Other statistical testing alternatives, albeit less robust ones for our dataset, lead to an identical conclusion (See *Supportive Information*, SI8). This leads to the conclusion that there are no statistically significant differences in mean sub-lethal Cd tolerance among the 10 natural pond populations.

3.3. Comparison of tolerance of natural pond populations with 'population' of laboratory clones

None of the 10 field populations showed a significantly different mean Cd tolerance at around 4 $\mu\text{g/L}$ compared to the group of laboratory clones (i.e., the mean of the collection of seven laboratory clones). This conclusion is based on the result of t -tests for independent samples with p -values between 0.159 and 0.963 or of Mann–Whitney U tests (in case of non-normality or unequal variance) with p -values between 0.135 and 0.735 (see *Supportive Information*, Table S9 for details).

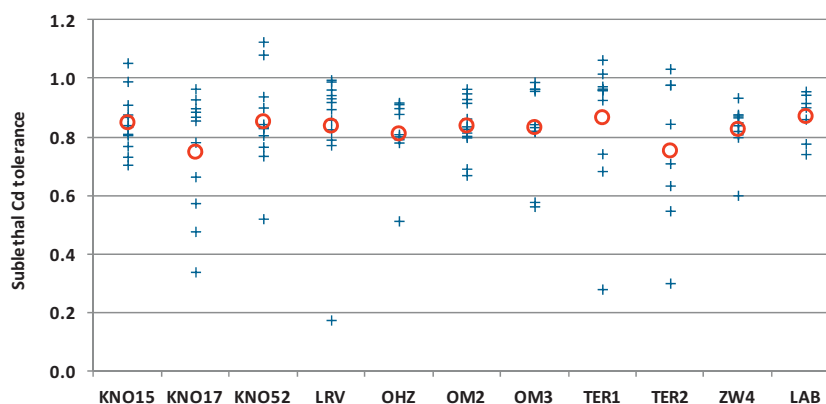


Fig. 2. Sub-lethal Cd tolerance in 10 natural populations (KNO15 to ZW4) and in a collection of 7 isoclonal laboratory populations from ecotoxicology laboratories (LAB). Crosses represent means of each clone within a population. Circles are the population means (mean of clone means). Population means are available in *Supportive Information* (Table S5). Cd tolerance is calculated as r_m in the Cd treatment divided by r_m in the control treatment (Eq. (2)).

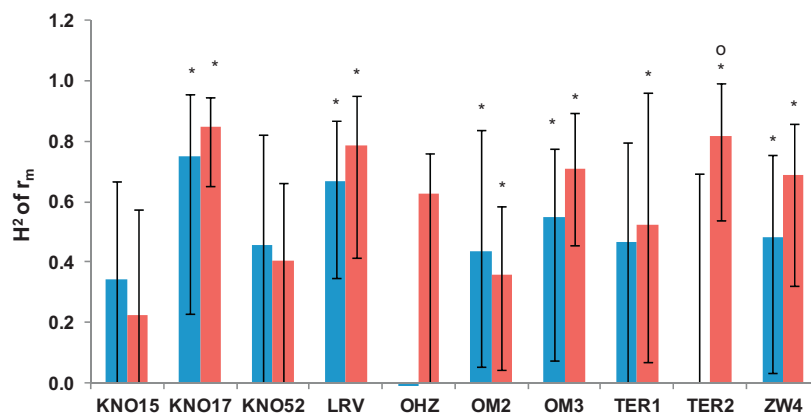


Fig. 3. Median broad sense heritabilities (H^2) of intrinsic rates of increase (r_m) under control (blue) and Cd environments (red) in 10 natural *D. magna* populations. Error bars indicate 90% confidence interval. Values of H^2 and confidence limits are available in *Supportive Information* (Table S10). An asterisk (*) indicates $H^2 > 0$ in this population \times environment combination. An open circle (o) indicates that H^2 is significantly different between the control and the Cd environment within a population (only the case for TER2).

3.4. Heritability of r_m and Cd tolerance of 10 natural pond populations

A broad range of median estimates of H^2 was observed across populations for r_m under control and Cd exposure (Fig. 3) and for Cd tolerance (Fig. 4) (see *Supportive Information*, Table S10 for all values). In the control $H^2(r_m)$ varied between -0.59 (OHZ) and 0.75 (KNO17) across all ten investigated populations, with a mean (\pm S.D.) of 0.36 ± 0.39 . In the Cd treatment $H^2(r_m)$ varied between 0.23 (KNO15) and 0.85 (KNO17), with a mean of 0.60 ± 0.21 . $H^2(r_m)$ in the Cd environment was significantly higher than 0 for seven populations, and for five of those populations $H^2(r_m)$ was also significantly higher than 0 in the control environment (with $H^2(r_m)$ between 0.44 and 0.75) (Fig. 3, Table S10). For most populations (except OHZ and TER2) median estimates of H^2 were similar between the control and Cd environment (Fig. 3). Considering all populations together, H^2 shows no significant trend of being higher in the Cd environment than in the control (Wilcoxon matched pairs test, $p = 0.074$, $n = 10$). When populations are considered separately, only in the TER2 population the H^2 in the Cd environment is significantly higher than in the control (non-parametric bootstrapping, $p = 0.011$). Pair-wise comparisons of the H^2 values between populations but within the same environment, revealed significant differences for 8 of 55 possible population pairs in the control environment (OHZ differs from 8 other populations except TER2) and

for 4 population pairs in the Cd environment (i.e., KNO15-KNO17, KNO15-TER2, KNO17-KNO52, and KNO17-OM2) (All comparisons performed with non-parametric bootstrapping, $p < 0.05$, see *Supportive Information*, Table S11 for all pair-wise p -values.).

Across all ten populations, H^2 of Cd tolerance varied between 0.11 (KNO52) and 0.81 (TER2), with an average of 0.49 ± 0.26 . We found that H^2 of Cd tolerance was significantly higher than 0 in 5 of these 10 populations (with H^2 (Cd-tolerance) between 0.48 and 0.80) (Fig. 2). Each of these five populations also had $H^2(r_m)$ significantly higher than 0 in the Cd environment. Pair-wise population comparisons revealed significant differences of H^2 (tolerance) between six population-pairs, i.e. KNO15-KNO17, KNO17-KNO52, KNO17-OM2, KNO52-OM3, KNO52-TER2, and OM2-TER2 (Non-parametric bootstrapping, $p < 0.05$, see *Supportive Information*, Table S11 for all pair-wise p -values.).

4. Discussion

The cyclical parthenogen *D. magna* is one of the most frequently-used model organisms in ecotoxicology and for risk assessment of chemicals. Yet, genetically determined variation of chemical tolerance traits among parthenogenetically (asexually) reproducing clones complicates predictions of ecologically realistic responses of natural *D. magna* populations from results of typical laboratory ecotoxicity tests, which are usually conducted with a single laboratory

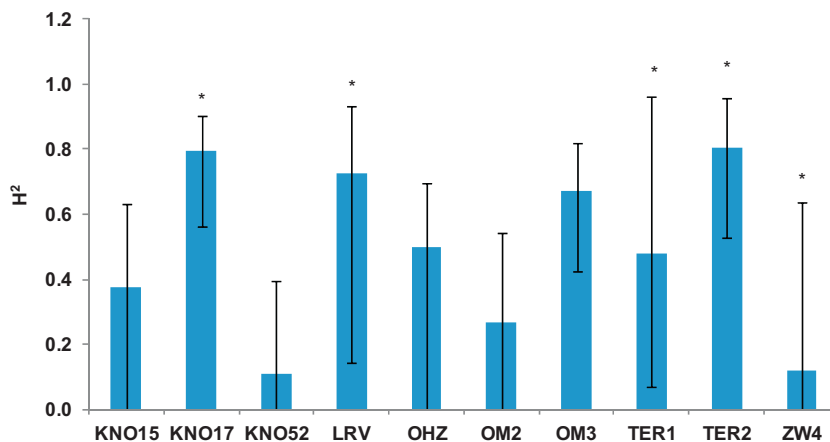


Fig. 4. Median broad sense heritabilities H^2 of sub-lethal Cd tolerance in 10 natural *D. magna* populations. Values of H^2 and 90% confidence limits are available in *Supportive Information* (Table S10). An asterisk (*) indicates $H^2 > 0$ and, thus, that there is a significant genetic component to Cd tolerance and a significant evolutionary potential of this sub-lethal tolerance trait in this population.

clone (Barata et al., 2002a; Messiaen et al., 2010). In this context, our study provides three pieces of information that are important in the context of the implications of this issue for ecologically relevant risk assessment, each of which will be discussed below.

First, we found a 14-fold difference in 21d-EC₁₀ values of Cd among 7 *D. magna* laboratory clones maintained in ecotoxicity testing laboratories across Europe, with 21d-EC₁₀ values of these clones ranging between 0.9 and 11 µg Cd/L (Table 2). This observed inter-clonal variation is in line with and even slightly larger than the earlier-reported 4–10-fold inter-clonal variation of sub-lethal toxicity of a variety of substances (Cd, Cu, NaBr, fluoranthene, dichloro-aniline, parathion, λ-cyhalotrin) among laboratory clones of *D. magna* based on feeding or reproductive traits (Baird et al., 1990; Soares et al., 1992; Barata et al., 2000a,b). Our finding reinforces the statement of Barata et al. (2002a) that the conclusions from a risk assessment (or the derivation of water quality criteria) for a given chemical, based on chronic ecotoxicity test data with only one *D. magna* clone, may be strongly dependent on the clone that was tested. Although risk assessment using data from a single clone is a crucial first step, our and earlier observations provide strong arguments for the implementation of multiple clone testing.

Second, we found no significant differences of the sub-lethal Cd tolerance at 4.6 µg Cd/L among 10 Cd-naïve field populations, with reductions of r_m in all populations ranging between 13% and 25%, relative to the control (Fig. 2, Table S5). This result supports and extends earlier findings of small differences (<1.6 fold) in reproductive EC₁₀-values for Cd and λ-cyhalotrin between 3 natural *D. magna* populations (Barata et al., 2002a) and of small, non-significant differences in effects of Cd on fitness among 4 natural *D. magna* populations exposed to a sub-lethal Cd concentrations between 0.5 and 2 µg/L (Barata et al., 2002b). In both these earlier studies and ours, all populations were collected from habitats with no indication of current or historical Cd pollution (see Table 1, see Section 3.2). As opposed to what has been reported for sub-lethal toxicity, more and a wider variety of results have been reported for exposures of naïve *D. magna* populations to lethal chemical concentrations. On the one hand, small (1.6-fold) and insignificant differences of acute copper and zinc toxicity (measured as median effective concentrations) have been found among two and three natural *D. magna* populations, respectively (Bossuyt et al., 2004; Muyssen et al., 2005). On the other hand, other studies did find significant inter-population differences of lethal toxicity. Barata et al. (2002b) found significant inter-population differences in longevity, ranging between about 3 and 10 days, among 4 naïve *D. magna* populations when exposed to 10 µg Cd/L. Coors et al. (2009) found 2.1-fold differences in acute 48h-EC₅₀s of K₂Cr₂O₇ among 10 *D. magna* pond populations, and these were also significant. Finding the explanation for this difference in among-population observation between lethal and sub-lethal toxicity is an interesting avenue for further research.

In addition, we found that none of our ten study populations appeared to show a different mean tolerance when compared with the mean tolerance of the group of 7 laboratory clones when exposed to the same Cd concentration of 4.6 µg Cd/L (Table S9). Collectively, this means that the mean sub-lethal tolerance of naïve natural populations to Cd (a measure of the *proximate* or *initial* relative response to Cd, see Section 1) could be relatively accurately predicted by the mean tolerance of a collection of only a hand-full of often-used ecotoxicology laboratory clones. If this finding would be confirmed for a broader range of toxicants, it could clearly aid in the improvement of lab-to-field extrapolation in risk assessment practice, at least for naïve populations. This would be an important improvement of the current situation, where risk assessment continues to be dominated by largely arbitrary assessment factors (e.g., EC, 2011a,b; ECHA, 2008).

Third, while all populations exhibit a similar mean tolerance and are thus all predicted to experience a similar *proximate* reduction of population mean r_m when exposed to Cd, the within-population genetic variation of sub-lethal Cd-tolerance traits (i.e., r_m (Cd) and tolerance), measured as broad-sense heritability (H^2), does show significant differences among some of these populations (Figs. 3 and 4, Table S11). Thus, several populations show genetic variation in Cd tolerance, and the degree they do so differs among populations. This finding with Cd as the stressor is in line with Ebert et al. (1998), who reported – for a set of four *D. magna* populations – significant within-population genetic variation of several traits that are indicative of tolerance to a bacterial parasite stressor. The finding of differences in H^2 values among populations predicts different capacities for micro-evolutionary responses among populations upon exposure to Cd. Based on whether or not H^2 values of r_m (Cd) or of relative Cd-tolerance were significantly greater than zero, we can broadly classify populations in three groups (Table S10). Below, we describe expected micro-evolutionary effects of Cd for each of these in terms of the potential for *resistance* development (as defined in Section 1) and changes in genetic (clonal) composition.

A first group of populations (KNO17, LRV, OM3, TER1, TER2) all exhibit significant genetic variation of Cd-tolerance (Fig. 4, Table S10), with H^2 values between 0.48 and 0.81 (mean ± S.D.: 0.70 ± 0.13). This means that in group I, inter-individual variation of relative Cd-tolerance is significantly, and to a large extent (48–81%) determined by genetic factors and that the relative Cd-tolerance trait (as defined in Eq. (2)) can respond to selection (Klerks et al., 2011; Visscher et al., 2008). All these populations also exhibit a significant H^2 of r_m (Cd), with H^2 values between 0.52 and 0.85 (mean ± S.D.: 0.74 ± 0.13) (Fig. 3, Table S10), and their clone means for Cd-tolerance are highly significantly and positively correlated with the clone means for r_m (Cd) (product-moment correlations $r = 0.91–0.98$, $p < 0.001$). As a result, multi-generational exposure of these populations to Cd is expected to lead to increasing frequencies of clones with higher Cd-tolerance and, thus to an increasing mean population Cd-tolerance (i.e., increased resistance *sensu* Morgan et al., 2007). Overall, this first group of populations also exhibits a higher H^2 of r_m (Cd) compared to H^2 of r_m (control) (Wilcoxon matched-pairs test $p = 0.043$). This suggests, that these populations may show more rapid changes in genetic (clonal) composition in the presence than in the absence of Cd and that they may also show faster clonal erosion (reduction of clonal diversity) in the presence than in the absence of Cd (Vanoverbeke and De Meester, 2010; see also Prugnolle et al., 2005). Barata et al. (2002b) also reported significant H^2 of sub-lethal Cd-tolerance and of fitness under 0.5–2.0 µg Cd/L exposure in two natural, naïve *D. magna* populations and also concluded that there was a strong potential in these populations to select for more Cd-tolerant genotypes (clones) under Cd exposure, and thus to evolve resistance to Cd stress.

In contrast to Barata et al. (2002b), however, in our larger survey of populations we have also observed populations that do show different characteristics (Figs. 3 and 4, Table S10). Indeed, a second group of populations in our study (comprising ZW4 and OM2) exhibit no significant H^2 of Cd-tolerance, but they do show significant H^2 of r_m (Cd) and of r_m (control) (Figs. 3 and 4, Table S10). This implies that these two populations have the capacity to show micro-evolutionary responses in r_m both in the Cd and the control environment, but that development of *resistance* (increasing frequency of more Cd-tolerant clones) under Cd exposure is not expected. In addition, changes in genetic composition and reduction in clonal diversity in these two populations are expected to take place at a similar rate, based on similar median estimates and non-significant differences of H^2 (r_m) between the control or the Cd environment (Fig. 3, Tables S10 and S11). A third group of populations (KNO15, KNO52, and OHZ) show no significant heritabilities

in any of the traits (Figs. 3 and 4, Table S10). Our results therefore indicate that these population have no or at most very low evolutionary potential to genetically respond to Cd exposure.

Collectively, while all naïve populations studied showed similar mean *initial* (proximate) sub-lethal tolerance to Cd in our experiments, our results also indicate that they may differ substantially in their capacity to evolutionarily respond to long-term, multi-generational exposure to Cd, in terms of the rate of change in clonal composition and/or in terms of the development of genetically determined *resistance*. Overall, in half of the studied populations (i.e. those in the second and third group of populations, as discussed in the paragraph above) the capacity to develop increased Cd tolerance under long-term Cd stress is weak. Together with the observation that the mean *initial* Cd tolerance of all field populations is similar to the mean tolerance of 7 laboratory clones, this means that the long-term effect of Cd (relative to a control) in these five populations could be relatively accurately predicted from the mean tolerance of only a handful of laboratory clones. However, this is clearly not the case for the other half of the studied populations that do show a strong evolutionary capacity to develop increased tolerance. In addition, it is noted that the observed H^2 values of tolerance cover a large portion of the theoretically possible range of H^2 values, i.e. they range from non-significantly different from zero (for five populations) to between 0.41 and 0.81 (for the other five populations). It will therefore be difficult or even impossible to predict the long-term, micro-evolutionary response of unstudied naïve populations from data on tolerance (and genetic variation thereof) obtained with a sample of other populations. Thus, we suggest that other approaches will likely be needed to forecast long-term effects of chemical on field populations, such as the development of predictive models based on the combination of long-term time-series of population-genomic (DNA sequences) and tolerance data, e.g. as they are archived in dormant egg banks (see Orsini et al., 2013 for details).

5. Conclusions

We observed that 21d-EC₁₀s of 7 often-used laboratory clones of *D. magna* varied 14-fold (between 0.9 and 11 µgCd/L, with a geometric mean of 3.7 µg/L). This indicates that risk assessment of chemicals and derivation of EQS should preferably be based on ecotoxicity test results from multiple clones rather than from single clones. Further, when exposed to a concentration of 4.4 µg Cd/L, which is close to the geometric mean EC₁₀, ten naïve natural pond populations of *D. magna*, collected from a wide variety of habitats, did not exhibit any significant difference in their mean sub-lethal, reproductive tolerance (with observed mean tolerances ranging between 0.75 and 0.91). Furthermore, the tolerance of none of these ten populations differed from the mean tolerance of the 7 laboratory clones (which was 0.87). Together, this means that the mean *initial* (proximate) response of an unstudied naïve natural *D. magna* population to Cd exposure (relative to a control) can be predicted relatively accurately from the mean tolerance of another naïve natural population or from the mean tolerance of only a handful of investigated laboratory clones. This makes risk assessment using a multiple clone approach feasible and relevant, at least to predict initial effects of pollutants. For longer term effects that also involve the possibility of micro-evolutionary adaptation, we find that predictions will be much more difficult or even impossible, because evolutionary potential was shown to differ substantially among populations. Thus, evolutionary potential should preferably be measured for each focal study population separately and different approaches for forecasting micro-evolutionary responses of naïve natural populations to chemical exposure should be explored, such as the development of predictive models based on the combination of long-term genomic and tolerance time-series data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2013.10.016>.

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